

Basic Investigations

Astragaloside IV Regulates Expression of ATP-sensitive Potassium Channel Subunits after Ischemia-reperfusion in Rat Ventricular Cardiomyocytes

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Objective: Astragaloside IV (AsIV) is the major effective component extracted from the Chinese herb *Astragalus membranaceus*, which has been widely used to treat cardiovascular disease. Recent studies have shown that AsIV can potentially protect the heart from myocardial ischemic injury, but the mechanisms of action are unknown. ATP-sensitive potassium (K_{ATP}) channels are activated during ischemia and exert a compensatory protective effect on cardiomyocytes. We therefore examined the effects of AsIV on K_{ATP} channel currents and channel expression in isolated rat ventricular cardiomyocytes after ischemia-reperfusion injury.

Methods: Forty Wistar rats were divided into five groups: control group, ischemia-reperfusion (IP) group, IP + glibenclamide group, IP + pinacidil group and IP + AsIV group. The ischemia-reperfusion injury model was established in enzymatically isolated ventricular cardiomyocytes by perfusion with calcium-free Tyrode solution for 10 min, arrest for 30 min, and reperfusion for 45 min. The different drugs were applied for 10–15 min, and the K_{ATP} channel current ($I_{K_{ATP}}$) was recorded with voltage-clamp mode by whole-cell patch-clamp technique. Protein and mRNA expression of the K_{ATP} channel subunits Kir6.1, Kir6.2, SUR2A and SUR2B was quantified using western blotting and real-time PCR.

Results: The K_{ATP} current in IP group was significantly greater than that in control group (211.45 ± 33.67 vs 83.51 ± 23.67 pA; $P < 0.01$). Glibenclamide ($10 \mu\text{mol/L}$) blocked K_{ATP} currents, whereas both AsIV (1 mg/L) and the known channel opener pinacidil ($50 \mu\text{mol/L}$) significantly increased $I_{K_{ATP}}$ ($P < 0.05$). Consistent with this, AsIV significantly up-regulated protein and mRNA expression of Kir6.1, Kir6.2, SUR2A, SUR2B ($P < 0.01$ vs IP group).

Conclusion: The protective effects of AsIV in ischemia-reperfusion injury may be related to the up-regulation of several K_{ATP} channel subunits and facilitation of K_{ATP} currents.

Keywords: Astragaloside IV; pinacidil; myocardial ischemia-reperfusion; K_{ATP} channels

Ischemic heart disease (coronary heart disease) remains a major threat to human health. Ischemia-reperfusion may activate some ion channels that cannot open under normal physiological conditions. One such channel is the ATP-sensitive potassium (K_{ATP}) channel, which opens in response to a drop in cellular ATP levels and a decrease in the ATP/ADP ratio during ischemia. Channel activation facilitates potassium ion efflux, encouraging hyperpolarization and action potential repolarization. The resulting shortening of the action potential duration decreases the total influx of sodium and calcium. This inhibition of calcium influx alleviates overloading of intracellular calcium, weakens myocardial contraction force and reduces myocardial oxygen consumption. Thus, the opening of K_{ATP} channels plays an active role in protecting the heart against myocardial ischemia or hypoxia.

Astragaloside IV (AsIV) is a major active component of the native Chinese herb *Astragalus membranaceus*, which has been widely used for the treatment of cardiovascular disease in China. Recent studies have shown that AsIV may play a potential role in protecting the heart from myocardial ischemia. The mechanism of

action may involve antioxidative and nitric oxide-inducing properties, reduction of $[\text{Ca}^{2+}]_i$ and sarcoplasmic reticulum (SR) Ca^{2+} load, enhanced free radical removal, and decreased lipid peroxidation.^{1,2} AsIV may also improve cardiac function in rats after ischemia-reperfusion injury,³ potentially due to prevention of $[\text{Ca}^{2+}]_i$ overload through improvements in energy metabolism, scavenging of oxygen free radicals, and inhibition of free radical production in the ischemic myocardium. The aim of this study was to investigate the effects of AsIV on K_{ATP} channel currents and subunit

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expression in rat ventricular myocytes after ischemia-reperfusion, to explore the mechanisms of AsIV action and provide experimental data for the development of new drugs.

METHODS

Animals and Grouping

Forty male Wistar rats (specific pathogen-free grade, provided by Shanghai Slac Laboratory Animal Company, Shanghai, China), weighing 200–300 g, were housed on a 12:12 light/dark cycle in the experimental animal center of Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine. Water and food were freely available. All experimental procedures were approved by the Animal Care Committee of Shanghai University of Traditional Chinese Medicine and were in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. The animals were randomly divided into five groups: control group, ischemia/reperfusion (IP) group, IP + glibenclamide (Gli) group, IP + pinacidil (Pina) group, and IP + AsIV group.

Solution and Reagents

Calcium-free Tyrode solution consisted of (in mmol/L) NaCl 137.0, KCl 5.0, MgSO₄ 1.2, NaH₂PO₄ 0.5, glucose 10.0, and HEPES 10.0 (pH was adjusted to 7.35–7.4 with NaOH). Tyrode solution consisted of (in mmol/L) NaCl 137.0, CaCl₂ 1.8, KCl 5.0, MgSO₄ 1.2, NaH₂PO₄ 0.5, glucose 10.0, and HEPES 10.0 (pH was adjusted to 7.35–7.4 with NaOH). KB solution consisted of (in mmol/L) KOH 70.0, KCl 40.0, KH₂PO₄ 20.0, L-glutamic acid 50.0, taurine 20.0, EGTA 0.5, HEPES 10.0, and glucose 10.0 (pH was adjusted to 7.35–7.4 with KOH). The extracellular solution used for whole-cell recording consisted of (in mmol/L) NaCl 137.0, CaCl₂ 1.2, KCl 5.0, MgSO₄ 1.2, NaH₂PO₄ 0.5, glucose 10.0, and HEPES 10.0 (pH was adjusted to 7.35–7.4 with NaOH).

Primary antibodies: Kir6.1, Kir6.2, SUR2A, SUR2B, β -actin and rabbit anti-mouse antibody were purchased from Wuhan Boster Biological Technology Co., Ltd. Wuhan, China. Secondary antibodies: goat anti-rabbit IgG HRP was produced by Rockland Immunochemicals Inc. (Gilbertsville, PA, USA). All antibodies were diluted 1:2000 in PBS-T before analysis. The primers were synthesized by Shanghai Invitrogen Co., Ltd. (Shanghai, China). Pinacidil and glibenclamide were provided by Sigma-Aldrich Co. LLC (St Louis, MO, USA). Astragaloside IV was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Preparation of the Ischemia-reperfusion Model in Ventricular Cardiomyocytes

The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight). The heart was excised in oxygen-saturated Tyrode solution at 4 °C and was given retrograde perfusion in a non-recirculating Langendorff apparatus at constant

pressure. All solutions were saturated with 95% O₂/5% CO₂ and the perfusion system was kept at a temperature of 37 °C with a flow velocity of 8–10 mL/min. The isolated heart was first infused for 10 min with calcium-free Tyrode solution and then subjected to a 20 min global no-flow ischemia followed by a 45 min infusion of calcium-free Tyrode solution, and finally a 30 min infusion of low-calcium Tyrode solution containing 0.5 g/L of collagenase (Sigma-Aldrich Co. LLC, St Louis, MO, USA) and 20 μ mol/L Ca²⁺. The ventricle was cut into small pieces in KB solution and incubated for 10 min. The dispersed cells were harvested by filtering with a 200- μ m nylon mesh. The isolated ventricular myocytes from the second and third incubations were washed with Medium 199 containing 10% calf serum by low speed centrifugation before they were used for experiments. When the calcium concentration of the filtrate returned to 1.25 mmol/L at room temperature, 70–80% rod-shaped calcium-resistant myocardial cells retaining normal physiologic and metabolic functions were acquired.

Whole-cell Patch-clamp Recordings

K_{ATP} currents were recorded after 10–15 min of bath-application of the different drugs (glibenclamide 10 μ mol/L, pinacidil 50 μ mol/L and astragaloside IV (1 mg/L). Recordings were made with standard whole-cell patch clamp techniques. Membrane currents were recorded and analyzed with an Axoclamp 200B amplifier in conjunction with the pClamp 6.0 software and the analog/digital interface (Axon Instruments, Union City, CA, USA). Voltage commands were delivered using Clampex 8.2 running on an IBM-compatible computer.

Real-time Quantitative PCR Assays

Total RNA was extracted from isolated ventricular cardiomyocytes treated with different drugs using a TRIzol kit, and subjected to one-step real-time quantitative PCR. The reverse transcription reaction was performed using 50 ng of total RNA that was reverse transcribed into cDNA using oligo (dT) primer, then amplified for 40 cycles using two oligonucleotide (ODN) primers (Kir 6.1: 5'-GAGTGA-ACTGTCGCACCAGA-3' and 5'-CGATCACCAGAA-CTCAGCAA-3'; Kir6.2: 5'-CGATCACCAGAACTCA-GCAA-3' and 5'-GATGGGGACAAAACGCTG-3'; SUR2A: 5'-GGAGCAATCCAGACCAAGAT-3' and 5'-AGCCAGCAGAATGATGACAG-3'; SUR2B: 5'-ACCTGCTCCAGCACAAGAAT-3' and 5'-TCTCTT-CATCACAATGACCAGG-3'). Each PCR cycle was carried out for 30 s at 95 °C, 5 s at 95 °C and 30 s at 60 °C. Serial dilutions of cDNA were used to construct the standard curve. Each data point was repeated three times. Quantitative values were obtained from the threshold PCR cycle number (Ct) at which the increase of signal was associated with an exponential growth of PCR product after it started to be detected. The relative expression levels of the target gene were calculated according to the equation $\Delta\Delta CT = (CT_{\text{Target}} - CT_{\text{Actin}})_{\text{Time x}} - (CT_{\text{Target}} - CT_{\text{Actin}})_{\text{Time 0}}$.

Western Blotting Analysis

Cells were rinsed twice with ice-cold PBS following treatment and scraped into ice-cold buffer containing 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 50 mmol/L NaF, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L DTT, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L leupeptin, and 5 mg/mL aprotinin. After lysis on ice for 30 min, the extracts were centrifuged for 15 min at 14000 g at 4 °C. The protein concentration of the supernatant was measured, and 20 µg protein samples of cell lysate were mixed (1:1) with Laemmli sample buffer and incubated at 95 °C for 5 min. Proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in 7.5% acrylamide gels. The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by Western blotting using specific antibodies. Membranes were blocked in 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20 containing 5% non-fat dry milk, followed by incubation with primary antibody. Membranes were washed 3 times and incubated with the appropriate horseradish peroxidase conjugated secondary antibody for 2 h. The immunocomplexes were visualized by chemiluminescence with the Phototope western blot detection system. The images were digitalized using the Gel Imaging System (Alpha Innotech Corp, USA). Band densitometry was carried out using NIH Image software. The expression levels of Kir6.1, Kir6.2, SUR2A, and SUR2B were expressed by ratios of these proteins to β-actin (internal standard).

Statistical analysis

All the data were expressed as mean±SD. Statistical analysis was carried out using Student's *t* test and one-way ANOVA by SPSS12.0 software (SPSS Inc.). A value of *P*<0.05 was considered to be statistically significant.

RESULTS

Effect of Astragaloside IV on $I_{K_{ATP}}$ in Rat Ventricular Cardiomyocytes

Under physiological conditions, K_{ATP} channels in ventricular cardiomyocytes are predominantly closed. Therefore, the membrane was clamped at -40 mV and subjected to a ramp from -100 to 80 mV to induce background currents. An outward current in the control

group was 83.51 ± 23.67 pA, whereas it was significantly increased in IP group (211.45 ± 33.67 pA; *P*<0.01 vs the control group). The outward current after ischemia-reperfusion was significantly blocked by glibenclamide (an antagonist of K_{ATP} channels), so this current was identified as $I_{K_{ATP}}$. In the presence of pinacidil (an agonist of K_{ATP} channels), the outward current increased to 317.87 ± 33.75 pA (*P*<0.01 vs IP group). Consistent with a K_{ATP} channel-mediated mechanism of AsIV, the IP + AsIV group showed a significantly increased outward current amplitude (406.22 ± 23.28 pA; *P*<0.01 vs IP group) (Table 1).

Figure 1 shows the outward current and voltage relationship. The current-voltage curve displayed an N shape, these voltage-dependent of currents were rising compared with the background currents, corresponding to the increased $I_{K_{ATP}}$.

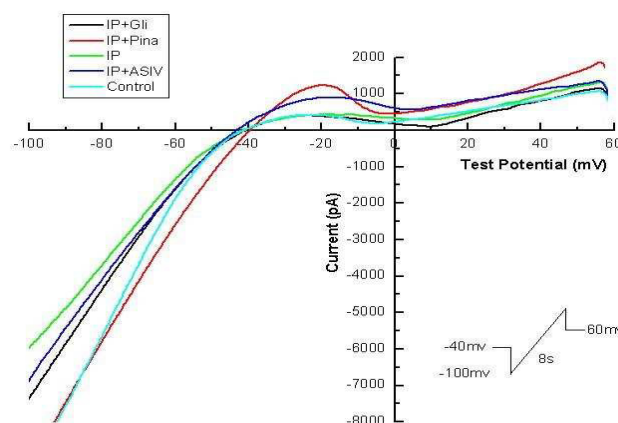


Figure 1. $I_{K_{ATP}}$ current-voltage curves of ventricular cardiomyocytes. IP: ischemia/reperfusion; IP + Gli: IP + glibenclamide; IP + Pina: IP + pinacidil; IP + AsIV: IP + Astragaloside IV.

Effect of Astragaloside IV on the Expression of K_{ATP} Channel Subunit mRNA

Quantitative real-time PCR demonstrated that the relative mRNA expression of the K_{ATP} channel subunits SUR2A, Kir6.1, Kir6.2, SUR2B were significantly higher in the IP group than in the control group (*P*<0.01). Compared with the IP group, the subunit mRNA expression was significantly decreased in the IP + Gli group, and increased in the IP + AsIV and IP + Pin groups (Table 2).

Table 1. Effect of astragaloside IV on K_{ATP} currents of rat ventricular cardiomyocytes at 0 mV

Group	<i>n</i>	K_{ATP} current amplitude (pA)
Control	8	$83.51\pm23.67^{\Delta\Delta\Delta}$
IP	8	$211.45\pm33.67^{**\Delta\Delta}$
IP+Gli	8	$129.68\pm30.92^{*\Delta\Delta\Delta}$
IP+Pina	8	$317.87\pm33.75^{**\Delta\Delta}$
IP+ AsIV	8	$406.22\pm23.28^{**\Delta\Delta\Delta}$

Notes: IP: ischemia/reperfusion; IP + Gli: IP + glibenclamide; IP + Pina: IP + pinacidil; IP + AsIV: IP + Astragaloside IV. Data are presented as mean ± SD. **P*<0.05, ***P*<0.01 vs control group; Δ *P*<0.01 vs IP group; $\Delta\Delta$ *P*<0.01 vs IP+Pina group.

Table 2. Effect of astragaloside IV on expression of K_{ATP} channel subunit mRNA

Group	n	Kir6.1	Kir6.2	SUR2A	SUR2B
Control	8	1.33±0.28 ^{▲▲}	1.28±0.30 ^{▲▲}	0.96±0.15 ^{▲▲}	1.09±0.23 ^{▲▲}
IP	8	3.15±0.58 [*]	2.61±0.36 [*]	1.80±0.27 [*]	2.10±0.41 [*]
IP+Gli	8	1.58±0.30 ^{▲▲}	1.18±0.18 ^{▲▲}	1.05±0.15 ^{▲▲}	1.08±0.20 ^{▲▲}
IP+Pina	8	5.64±0.82 ^{*△}	3.80±0.55 ^{*△}	3.10±0.54 ^{*△}	3.48±0.71 ^{*△}
IP+ AsIV	8	6.61±0.95 ^{*△▲}	5.18±0.73 ^{*△▲}	4.05±0.65 ^{*△▲}	4.30±0.64 ^{*△▲}

Notes: IP: ischemia/reperfusion; IP + Gli: IP + glibenclamide; IP + Pina: IP + pinacidil; IP + AsIV: IP + Astragaloside IV. The data are presented as mean±SD. **P*<0.01 vs control group; [△]*P*<0.01 vs IP group; [▲]*P*<0.01 vs IP + Pina group.

Effect of Astragaloside IV on K_{ATP} Channel Subunit Protein expression

Western blotting showed that the SUR2A, Kir6.1 and Kir6.2 proteins were expressed in ventricular cardiomyocytes of the control group, while SUR2B was not detected. The expression of SUR2A, Kir6.1 and Kir6.2 proteins was increased in the IP group compared to the control group (*P*<0.05). In the IP + Gli group, the channel expression was inhibited, whereas both pinacidil and AsIV increased expression. AsIV caused a greater increase in K_{ATP} channel subunit expression than did pinacidil (*P*<0.05, Table 3, Figure 2).

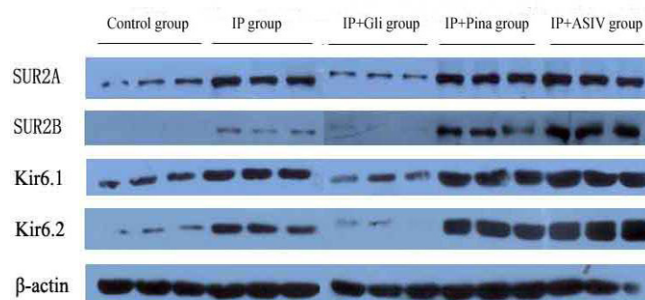


Figure 2. Expression of the K_{ATP} channel subunit proteins Kir6.1, Kir6.2, SUR2A, and SUR2B. IP: ischemia/reperfusion; IP + Gli: IP + glibenclamide; IP + Pina: IP + pinacidil; IP + AsIV: IP + Astragaloside IV.

Table 3. Effect of astragaloside IV on the relative expression of K_{ATP} channel subunit protein

Group	n	Kir6.1	Kir6.2	SUR2A	SUR2B
Control	8	0.33±0.17 ^{▲▲▲}	0.05±0.03 ^{▲▲▲}	0.12±0.13 ^{▲▲▲}	0
IP	8	1.11±0.071 [*]	0.62±0.16 [*]	0.75±0.12 ^{**}	0.0006±0.0010
IP+Gli	8	0.35±0.48 ^{▲▲▲}	0.02±0.03 ^{▲▲▲}	0.12±0.087 ^{▲▲▲}	0
IP+Pina	8	1.47±0.30 ^{**}	1.11±0.13 ^{**}	0.86±0.17 ^{**}	1.75±0.82 ^{**△▲}
IP+ AsIV	8	2.34±0.52 ^{**△▲▲}	1.85±0.98 ^{**△▲▲}	1.25±0.091 ^{**△▲▲}	4.85±1.14 ^{**△▲▲}

Notes: IP: ischemia/reperfusion; IP + Gli: IP+glibenclamide; IP + Pina: IP + pinacidil; IP + AsIV: IP + Astragaloside IV. Data are presented as mean ± SD. **P*<0.05, ***P*<0.01 vs control group; [△]*P*<0.05, [▲]*P*<0.01 vs IP group; ^{▲▲}*P*<0.05, ^{▲▲▲}*P*<0.01 vs IP + Pina group.

DISCUSSION

A decrease in cellular ATP during cardiac ischemia activates K_{ATP} channels that are silent under normal physiological conditions. As a result of the increased potassium current, the plateau phase of the action potential is shortened, which decreases total calcium influx. This in turn reduces calcium-related contraction and thereby energy consumption, thus providing a protective effect through negative feedback. During reperfusion, intracellular overloading of calcium triggers calcium-dependent phospholipid signaling, which leads to arachidonic acid (AA) metabolism. This causes production of oxygen free radicals, and the ischemic myocardium fails to take full advantage of oxygen, worsening the ischemic injury.

K_{ATP} channels were initially discovered by Noma in 1983, who used patch-clamping of cardiomyocytes of guinea pigs.^{4,5} The channels were classified as sarcolemmal K_{ATP} channels and mitochondrial K_{ATP} channels, and

these are thought to play important roles in myocardial ischemia/hypoxia, ischemic-reperfusion injury, and ischemic preconditioning.⁶⁻⁸ K_{ATP} channels can increase myocardial oxygen supply while decreasing oxygen consumption, thus improving cardiac function and myocardial energy metabolism, preserving the normal structure of the cells, regulating vascular tension, and changing ion distributions inside and outside the cardiac myocytes. These actions are shared by many endogenous vascular-active substances, such as adenosine, vasoactive intestinal peptide, and calcitonin gene related peptide, which are related to various signal molecules and signal transduction processes.⁹⁻¹¹

Myocardial protection research has increasingly focused on K_{ATP} channels as a mediator of endogenous and exogenous vascular dilation and contraction.^{12,13} Representative potassium channel openers, including cromakalin, diazoxide and pinacidil, may facilitate potassium ion efflux to induce membrane

hyperpolarization and repolarization, counteracting the opening of voltage-dependent calcium channels. The effects are mediated by inhibition of the formation of inositol triphosphate, reductions in vascular contraction factors that induce cytoplasmic release of Ca^{2+} , weakening of the sensitivity of organelles to Ca^{2+} , and acceleration of the clearing of intracellular Ca^{2+} by $\text{Na}^+-\text{Ca}^{2+}$ exchangers to decrease intracellular free calcium.¹⁴⁻¹⁷ When K_{ATP} channels are open, cardiac myocytes can be protected by facilitation of ATP synthesis or inhibition of calcium overloading. Further, protection can be achieved by the promotion of oxygen free radical production during ischemia and inhibition of oxygen free radical production during reperfusion. K_{ATP} antagonists, including glibenclamide and cibenzone, can block protective effect of K_{ATP} currents in the dog myocardium. In general, if an ion current was blocked by at least 100 $\mu\text{mol/L}$ glibenclamide, we considered this to be mediated by the K_{ATP} channel.¹⁸ In this study, the ischemia-reperfusion injury was induced by 30 min of ischemia, followed by 45 min of reperfusion. Under these ischemic and hypoxic conditions, I_{KATP} was significantly increased, and glibenclamide 10 $\mu\text{mol/L}$ could block most of the K_{ATP} current. In contrast, pinacidil could significantly increase I_{KATP} .

Some studies have indicated that AsIV can improve post-ischemic cardiac function, prevent overloading of calcium by stimulating calcium extrusion through calcium pumps, and increase the levels of superoxide dismutase to reduce accumulation of free radicals during myocardial ischemia, thus preventing lipid peroxidation injury.^{19,20} The present study indicates that AsIV could also open K_{ATP} channels, with similar efficacy to pinacidil.

The K_{ATP} channel is a tetramer containing the inward rectifier potassium channel protein Kir and the ATP-binding protein at a ratio of 1:1. The Kir protein forms an ion channel at the center of the tetramer, while the ATP-binding protein resides at the outskirts and determines the function of K_{ATP} . The main types of myocardial K_{ATP} channel subunits are SUR2A and Kir6.2, and vascular smooth muscle channels include SUR2B and Kir6.1.²¹⁻²⁵ In this study, the expression of SUR2A, Kir6.1 and Kir6.2 were observed in the cardiomyocytes, but there was almost no expression of SUR2B. This result is consistent with previous reports.^{26,27} In the IP group, which showed increased K_{ATP} currents, the expression of the K_{ATP} channel subunits was significantly increased, providing further support for a role of these channels in the ischemic myocardium. Pinacidil increased the expression of channel subunits to protect cardiomyocytes after ischemia-reperfusion injury, while glibenclamide could block this effect. Meanwhile, AsIV also significantly increased the expression of the K_{ATP} channel subunits, and the increase appeared to be larger

than the change observed after pinacidil. We suggest that AsIV may facilitate the opening of K_{ATP} channels.

In conclusion, AsIV is an opener of the K_{ATP} channel, and may thereby facilitate potassium ion efflux and induce membrane hyperpolarization and accelerate action potential repolarization, with resulting protective effects. The up-regulation of several protein subunits will also facilitate the opening of K_{ATP} channels, suggesting that AsIV is likely to have a cardioprotective effect after ischemia-reperfusion injury.

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